SPECIFICATION

GENE-MUTATED ANIMAL

Field of the Invention

This invention relates to a trans-genic animal. More specifically, the invention relates to a presenilin trans-genic animal with a transferred mutated presenilin gene causing human Alzheimer's disease.

Background of the Invention

Alzheimer's disease exhibits a symptom of progressive dementia. Its pathologic histology is characterized by emergence of a huge number of senile plaques in the brain and accumulation of neurofibrillary degenerations in neurons. The disease is neurodegenerative in which neurons are gradually leading to deciduation. Alzheimer's disease generally develops in old age and its prevalence is known to increase with aging. At present, a definitive treatment of Alzheimer's disease is impossible. Accordingly, in order to prepare for sharp increase of the old age population in the future, early developments of a method of therapeutic and preventive treatment of Alzheimer's disease and an effective medicament for preventive and therapeutic treatment of the disease are desired.

Senile plaque is a deposit outside neurons which contains various ingredients, and whose main ingredient is a peptide consisting of 39-42 amino acid residues called amyloid β protein (Aβ). Amyloid precursor protein (APP) is cleaved by proteases tentatively named β secretase and γ secretase to produce amyloid β. In the senile plaque, the amyloid β deposits as a rigid construct having β sheet structure. The senile plaque is first formed as a "stain-like" deposition called as a diffuse senile plaque. At this stage, neurodegeneration has not yet occurred. It is considered that, as the diffuse senile plaque becomes a more rigid deposition, the degeneration or deciduation of neurocytes occurs, which results in the onset of symptoms of Alzheimer's disease such as dementia. There are Aβ40 consisting of 40 amino acid residues and Aβ42 consisting of 42 amino acid residues as main amyloid β. Most of amyloid β generated by cells is Aβ40, and only a little amount of Aβ42 exists.

However, Aβ 42 has higher aggregation properties, and therefore, Aβ 42 is considered to have a more significant role than Aβ 40 in the formation of senile plaque (Tamaoka, Naika (Internal Medicine), Vol. 77, P843, 1996).

In Alzheimer's disease, familial onsets are observed which exhibit an autosomal dominant inheritance. A gene first identified as a causal gene of the familial onset of Alzheimer's disease in 1991 is a mutant of APP, a gene located on chromosome 21 in which amino acid residue at position 717 is mutated from valine to isoleucine (Goate A. et al., Nature, Vol. 349, P704, 1991).

Other mutants of APP as causes of Alzheimer's disease were found such as those where said amino acid residue at position 717 is mutated to phenylalanine (Murrell J. et al., Science, Vol. 254, P97, 1991); where the amino acid residue at the same position is mutated to glycin (Chartier, Harlin et al., Nature, Vol. 353, P844, 1991); where two amino acid residues at positions 670 and 671 are mutated from lysine-methionine to asparagine-leucine (Mullan M. et al., Nature Genet., Vol. 1, P345, 1992); and where amino acid residue at position 692 is mutated from alanine to glycin (Hendrisk L. et al., Nature Genet., Vol. 1, P218, 1992) and the like.

Apolipoprotein E (apo E) was reported in 1993 as a causal factor or a risk factor of the familial Alzheimer's disease. Persons with Alzheimer's disease were found to have apoE4, in which the amino acid residue at position 112 is arginine and the amino acid residue at position 158 is arginine, at a significantly higher rate than healthy persons among isomers of apoE whose genes are located on chromosome 19 (Corder E. H. et al., Science, Vole 261, P921, 1993).

After then, a mutant of the gene "presenilin-1" (PS-1, initially called as S182) being located on chromosome 14 (Sherrington R. et al., Nature, Vol. 375, P754, 1995) and a mutant of the gene "presenilin-2" (PS-2, initially called as E5-1 or STM-2) being located on chromosome 1 (Sherrington R. et al., Nature, Vol. 375, P754, 1995) were found as new causal genes for Alzheimer's disease in 1995 (in the specification, each gene is called as "presenilin-1 gene" and "presenilin-2 gene", respectively, and each gene product is called as "presenilin-1 protein" and "presenilin-2 protein", or "PS-1" and "PS-2", respectively.)

Presenilin-1 protein and presenilin-2 protein consisting respectively of 467 and 448 amino acid residues have a seven (or eight)-fold transmembrane primary

structure, and accordingly, they are presumably present as membrane proteins. Homology of the two proteins is high at amino acids level, i.e., 67% in total and 84% in the transmembrane domain alone. As for function of presenilin-1 protein, the protein is suggested to possibly have similar functions to nematode sel-12 protein or SPE-4 protein because of high homology to these proteins. SPE-4 protein participates in nematode spermatogenesis process and is considered to be involved in transport and storage of proteins.

Consequently, presenilin-1 protein is believed to participate possibly in processing of membrane proteins such as APP, axoplasmic transport, and fusion of membrane vesicle with membranes. The sel-12 was found as a gene which remedies an embryological abnormality caused by mutation of lin-12 which controls nematode development. The lin-12 is considered to be involved in intercellular signal transduction, and accordingly, presenilin-1 protein is also suggested to possibly participate in a certain step of intercellular signal transduction.

The first report on presenilin-1 protein describes that mutations causing the familial Alzheimer's disease are substitutions of amino acid residues at five positions. After this report, genes mutated at various sites were found from many families afflicted with familial Alzheimer's disease, which include OS-2 (isoleucine at position 213 is mutated to threonine) and OS-3 (valine at position 96 is mutated to phenylalanine), both reported by the present inventors (Kamino K. et al., Neurosci., Lett., Vol. 208, P195, 1996), and more than 40 types of amino acid substitutions have been known at more than 30 sites so far (Hardy. TINS, Vol. 20, P154, 1997).

At present, 70-80 % of the familial Alzheimer's disease is believed to be related to the mutation of presenilin-1 protein. Mutations at two sites have been reported as for presenilin-2 protein. As explained above, genetic analysis has proved that mutants of presenilin-1 and presenilin-2 proteins are deeply involved in the familial Alzheimer's disease.

Studies on mechanism how the mutants of presenilin-1 and presenilin-2 proteins cause the onset of Alzheimer's disease have also been progressed. It has been reported that Aβ40 is almost the same level as normal presenilin-1 and presenilin-2 proteins, whilst Aβ42 is highly increased as compared to normal presenilin-1 and presenilin-2 proteins in serum or a culture medium of dermal

fibroblasts from a patient with Alzheimer's disease having the aforementioned mutants (Scheuner D. et al.: Nature Med., Vol. 2, P864, 1996); in a culture medium of a cell line transformed by mutants of presenilin-1 protein and presenilin-2 protein (Xia W. et al.: J. Biol. Chem. Vol. 272, P7977, 1997; Borchelt D.R. et al.: Neuron, Vol. 17, P1005, 1996; Citron, M. et al.: Nature Med., Vol. 3, P67, 1997); and in the brain tissue of a patient with familial Alzheimer's disease having the mutant presenilin-1 protein (Lemere C.A. et al.: Nature Med., Vol. 2., P1146. 1996).

These reports show that the mutants of presenilin-1 protein and presenilin-2 protein, which cause the familial Alzheimer's disease, possibly trigger the onset of Alzheimer's disease by the increase of Aβ42 which is considered to play a significant role in the formation of senile plaque. A trans-genic mouse transferred with a gene encoding the mutant presenilin-1 protein was created (Duff K. et al.: Nature, Vol. 383, P710, 1996, Borchelt DR. et al.: Neuron, Vol. 17, P1005, 1996 and Citron M. et al.: Nature Med., Vol. 3, P67, 1997). It was reported that Aβ42 in the brain of the trans-genic mouse selectively increased. These results are strong supports of the possibility that mutants of presenilin-1 protein and presenilin-2 protein causing the familial Alzheimer's disease increase Aβ42 which possibly has significant roles in the formation of senile plaque, thereby develop Alzheimer's disease. However, no description is given about histological study of the mouse's brain in the above reports on the trans-genic mouse, which presumably due to no observation of remarkable histological change in the brain of the trans-genic mouse.

Generally, trans-genic animals are useful as a means of analyzing functions of a target gene *in vivo*. However, it is technically difficult to control the expression of a transferred gene quantitatively, tissue specifically, or time specifically during development. There is also a problem in that two different gene products are present as a mixture in the trans-genic animals since a gene inherently possessed by the animal still works for normal expression, and functions of a transferred gene cannot be sufficiently analyzed. Furthermore, when the transferred gene is subjected to particularly excessive expression, functions not inherently performed in vivo may appear in trans-genic animals, which results in a defect of possible confusion in analysis of constructed gene-mutated animals.

Apart from trans-genic animals, knockout animals may also be used as a

means of analyzing functions of a target gene. In a knockout animal, a target gene inherently possessed by the animal is artificially destroyed so as to be dysfunctional. A detailed analysis of knockout animals may reveal functions of a target gene in vivo. However, particular changes in knockout animals created as homozygote sometimes fails to appear, since the functions of the other gene products in the knockout animal may substitute for that of the destroyed gene products. Furthermore, there is also a problem in that an animal as homozygote may sometimes be lethal because the destroyed gene product is essential to the animal's development and growth, whilst thorough analysis of gene functions of an animal as viable heterozygote is practically impossible.

Disclosure of the Invention

An object of the present invention is to provide, for creation of an animal pathologic model of Alzheimer's disease, an animal as a pathological model whose pathologic conditions is closer to those of a patient with Alzheimer's disease, instead of a trans-genic animal having the aforementioned defects. More specifically, the object of the present invention is to provide a gene-mutated animal capable of expressing a mutant presenilin protein in the brain by transfer of a mutant of a presenilin gene which is believed to be a causal gene of Alzheimer's disease (a mutant presenilin gene) according to a homologous recombination technique. Further objects of the present invention are to provide a method of producing said gene-mutated animal; a plasmid useful for the aforementioned production method; and a method for evaluating a substance or an agent effective for preventive and/or therapeutic treatment of Alzheimer's disease using the aforementioned gene-mutated animal.

In order to reveal roles of presenilin-1 protein and mechanism of the onset of Alzheimer's disease by the mutation of presenilin-1 gene, the inventors of the present invention created a knockin mouse in which presenilin-1 gene inherently possessed by the mouse is replaced with the aforementioned presenilin-1 gene with OS-2 type mutation. As a result, the inventors found that the gene-mutated mouse successfully avoided the defects with the trans-genic mice and the knockout mice, and that the animal was useful for investigations of cause and pathology of familial Alzheimer's

disease caused by the mutant presenilin-1 gene. The inventors further continued the research, and achieved the present invention set out below.

The present invention thus provides a non-human gene-mutated animal having a mutant presenilin-1 gene, and more preferably, the invention provides a gene-mutated animal having a mutant presenilin-1 gene which comprises a DNA having a sequence encoding a presenilin-1 protein in which an amino acid in an amino acid sequence of a presenilin-1 protein is substituted with a different amino acid.

The present invention also provides:

a non-human gene-mutated animal having a mutant presenilin-1 gene which comprises a DNA having a sequence encoding a mutant presenilin-1 protein which has an amino acid sequence in which one or more amino acids at positions selected from the group consisting of amino acid numbers 79, 82, 96, 115, 120, 135, 139, 143, 146, 163, 209, 213, 231, 235, 246, 250, 260, 263, 264, 267, 269, 280, 285, 286, 290, 318, 384, 392, 410, 426, and 436 is substituted with different amino acid(s) in an amino acid sequences of a presenilin-1 protein, preferably a mouse-derived presenilin-1 protein; and

a non-human gene-mutated animal having a mutant presenilin-1 gene which comprises a DNA having a sequence encoding a mutant presenilin-1 protein which has one or more mutations selected from the group consisting of A79V, V82L, V96F, Y115H, Y115C, E120K, E120D, N135D, M139V, M139T, M139I, I143F, I143T, M146L, M146V, H163Y, H163R, G209V, I213T, A231T, A231V, L235P, A246E, L250S, A260V, C263R, P264L, P267S, R269G, R269G, R269H, E280A, E280G, A285V, L286V, S290C, E318G, G384A, L392V, C410Y, A426P and P436S in an amino acid sequence of a presenilin-1 protein, more preferably a mouse presenilin-1 protein (Each alphabet represents an amino acid expressed as a one-letter symbol, each number represents an amino acid number from the N-terminus of the presenilin-1 protein, and the descriptions mean that a wild-type amino acid shown in the left of the numerical figure is substituted with an amino acid shown in the right. In the specification, mutant presenilin-1 protein and mutant presenilin-2 protein are shown in the same manner.).

The present invention further provides a non-human gene-mutated animal

having a mutant presenilin-1 gene which comprises a DNA having a sequence encoding a mutant presenilin-1 protein in which isoleucine at position 213 of a presenilin-1 protein is substituted with an amino acid other than isoleucine, and a non-human gene-mutated animal having a mutant presenilin-1 gene which comprises a DNA having a sequence encoding a mutant presenilin-1 protein in which isoleucine at position 213 of a presenilin-1 protein is substituted with threonine.

According to preferred embodiments of the aforementioned inventions, there are provided:

the aforementioned gene-mutated animal having a mutant presenilin-1 gene wherein a DNA sequence encoding around an amino acid at position 213 in an amino acid sequence of a presenilin-1 protein is mutated to the following sequence:
5'-TGTGGTCGGGATGATMGCC ANC CACTGGAAAGGCCC-3'
wherein N represents a base other than T, M represents T or C, and the underlined bases encode the amino acid at position 213;

the aforementioned gene-mutated animal having a mutant presenilin-1 gene wherein a DNA sequence encoding around an amino acid at position 213 in an amino acid sequence of a presenilin-1 protein is mutated to the following sequence:
5'-TGTGGTCGGGATGATMGCC ANC CACTGGAAAGGCCC-3'
wherein N represents C, M represents T or C, and the underlined bases encode the amino acid at position 213; and

the aforementioned gene-mutated animal having a mutant presenilin-1 gene wherein a DNA sequence encoding around an amino acid at position 213 in an amino acid sequence of a presenilin-1 protein is mutated to the following sequence:
5'-TGTGGTCGGGATGATMGCC XYZ CACTGGAAAGGCCC-3'
wherein XYZ represents a codon as triplet bases which encodes an amino acids other than isoleucine, M represents T or C, and the underlined bases encode the amino acid at position 213.

From another aspect, the present invention provides a non-human gene-mutated animal having a mutant presenilin-2 gene which comprises a DNA having a sequence encoding a protein in which an amino acid at position 141 and/or 436 is substituted with a different amino acid in an amino acid sequence of a presenilin-2 protein. As a preferred embodiment of the invention, there is provided

the aforementioned non-human gene-mutated animal wherein the mutant presenilin-2 gene comprises a DNA having a sequence encoding a mutant presenilin-2 protein which contains a mutation of N141I and/or M239V in an amino acid sequence of a presenilin-2 protein.

As preferred embodiments of the aforementioned gene-mutated animals, the present invention provides the aforementioned gene-mutated animal wherein overexpression of amyloid β protein is caused by the mutant presenilin-1 gene and/or the mutant presenilin-2 gene; the aforementioned gene-mutated animal which can express the mutant presenilin protein and wherein the expression of said protein induces the production of amyloid β protein in an amount sufficient to form a progressive neural disease in a peripheral portion of the cerebral cortex of the brain of the animal; the aforementioned gene-mutated animal wherein the animal is a rodent, preferably a mouse; the aforementioned gene-mutated animal wherein the aforementioned mutant presenilin-1 gene and/or the aforementioned mutant presenilin-2 gene are transferred by homologous recombination; the aforementioned gene-mutated animal wherein amount of the amyloid protein expression in a brain tissue induced by the aforementioned presenilin-1 gene is sufficient to cause affected behavior in a memory learning test in comparison with a normal animal, and to induce abnormal neuropathy in a peripheral portion of the cerebral cortex of the hippocampus of the brain of the animal; and the non-human gene-mutated animal having a DNA which comprises a mutant preceilin-1 gene encoding a mutant preceilin-1 protein in which one or two or more amino acids is substituted with a different amino acid in the amino acid sequence of the presenilin-1 protein together with a DNA having a nucleotide sequence encoding a marker protein.

From further aspect, the present invention provides a plasmid comprising a DNA having a sequence of a mutant presenilin-1 gene wherein a DNA sequence encoding around an amino acid at position 213 of a presenilin-1 protein is the following sequence:

5'-TGTGGTCGGGATGATMGCC ANC CACTGGAAAGGCCC-3'

wherein N represents A, G, or C, M represents T or C, and the underlined bases encode an amino acid at position 213; and

a plasmid comprising a DNA having a sequence of a mutant presenilin-1 gene which

encodes a mutant presenilin-1 protein wherein an amino acid at position 213 is substituted with an amino acid other than isoleucine in an amino acid sequence of the presenilin-1 protein and a DNA sequence encoding around the amino acid at position 213 of presenilin-1 protein is the following sequence:

5'-TGTGGTCGGGATGATMGCC XYZ CACTGGAAAGGCCC-3'

wherein M represents T or C, XYZ denotes a codon as triplet bases encoding an amino acid other than isoleucine, and the underlined bases encode the amino acid at position 213. Additionally, the present invention also provides a chromosomal DNA containing exon 8 of a mutant presenilin-1 gene encoding a mutant presenilin-1 protein wherein an amino acid at position 213 is substituted with an amino acid other than isoleucine in an amino acid sequence of a presenilin-1 protein.

Furthermore, the present invention provides a plasmid comprising a DNA wherein a Sau3AI site is introduced into a nucleotide sequence comprising the whole or a mutated part of a cDNA or chromosomal DNA of a mutant presenilin-1 gene encoding a mutant presenilin-1 protein in which an amino acid at position 213 is substituted with an amino acid other than isoleucine in an amino acid sequence of presenilin-1 protein. Also provided are the aforementioned plasmid wherein the substitution of the amino acid is isoleucine at position 213 with threonine; and a plasmid comprising a DNA specified by the following nucleotide sequence:

5'-TGTGGTCGGGATGAMCGCCACCCACTGGAAAGGCCC-3'

wherein M represents T or C.

In addition to the above inventions, the present invention also provides a gene encoding a mouse mutant presenilin-1 protein wherein isoleucine at position 213 is substituted with an amino acid other than isoleucine in an amino acid sequence of a mouse presenilin-1 protein; and the aforementioned gene wherein the substitution is from isoleucine to threonine. Also provided are a plasmid comprising: (1) a gene encoding a mouse mutant presenilin-1 protein wherein isoleucine at position 213 is substituted with an amino acid other than isoleucine in an amino acid sequence of a mouse presenilin-1 protein; and (2) a neomycine expression unit flanked by loxPs; and the aforementioned plasmid wherein the substitution is from isoleucine to threonine (loxP has been disclosed in Japanese Patent Laid-Open Publication (Kohyo) No. 4-501501, page4).

From further aspect, the present invention provides an embryo introduced with a plasmid comprising a DNA represented by the nucleotide sequence: 5'-TGTGGTCGGGATGATMGCCACCCACTGGAAAGGCCC-3' wherein M represents T or C; an embryo obtained by homologous recombination using each of the aforementioned plasmids; and the aforementioned embryo derived from a mammalian rodent, more preferably from a mouse. The invention also provides a primary cell culture or subcultured cell obtained by isolating a cell from the aforementioned gene-mutated animal and culturing the cell by tissue culture; a method for producing a non-human gene-mutated animal wherein the method comprises the step of transferring a mutant presenilin-1 gene by homologous recombination into an embryo of an animal, wherein the mutant presenilin-1 gene is capable of expressing the mutant presenilin-1 and inducing production of amyloid β protein in an amount sufficient to form a progressive neural disease in a peripheral portion of the cerebral cortex of the brain; and the aforementioned production method wherein a mutant presenilin-1 protein can be expressed wherein isoleucine at position 213 is substituted with an amino acid other than isoleucine.

Additionally, the invention provides a method for evaluating a substance useful for therapeutic and/or preventive treatment of Alzheimer's disease which comprises the step of subjecting the aforementioned gene-mutated animal which is administered with a test substance to a comparison with the gene-mutated animal not administered with the test compound. A typical example of the method for evaluation includes a screening method. According to preferred embodiments of the invention, there are provided the aforementioned method for evaluation wherein the comparison is conducted by using a memory learning test; the aforementioned method for evaluation wherein the comparison is conducted by using a pathological test; the aforementioned method for evaluation wherein the comparison is conducted by a pathological test based on neuropathology in a peripheral portion of the cerebral cortex; the aforementioned method for evaluation wherein the comparison conducted by the pathological test based on neuropathology is a comparison of one or more items selected from the group consisting of suppression of decrease in overgrown gliosis in a peripheral portion of the cerebral cortex of the brain, suppression of decrease in uptake of 2-deoxyglucose in a peripheral portion of the cerebral cortex of the brain,

and suppression of decrease in availability of 2-deoxyglucose in the cerebral cortex of the brain; and the aforementioned method for evaluation wherein the comparison is conducted for one or more items selected from the group consisting of survival period of time, exploratory behavior and migratory behavior.

Still further, the present invention provides a method for evaluating a medicament for therapeutic and/or preventive treatment of Alzheimer's disease which comprises the step of culturing a primary cell culture or a subcultured cell *in vitro* in the presence of a test compound; a method for diagnosing Alzheimer's disease or a possibility of onset of Alzheimer's disease, which comprises the use of a partial nucleotide sequence of a mutant presenilin-1 gene encoding an OS-2 type mutant presenilin-1 protein; a substance useful for therapeutic and/or preventive treatment of Alzheimer's disease selected by each of the aforementioned evaluation methods; and a medicament for therapeutic and/or preventive treatment of Alzheimer's disease comprising the aforementioned substance as an active ingredient.

The present invention also provides a gene-mutated animal having a mutant presenilin gene and a gene encoding a mutant amyloid precursor protein, wherein the animal is a hybrid animal or its progeny which is produced by mating the aforementioned gene-mutated animal with an animal having a gene encoding a mutant protein of the amyloid precursor protein and a high productivity of amyloid β protein, and more preferably the animal is a hybrid mouse or its progeny which is produced by the mating or which is born as a result of the mating. According to a preferred embodiment of the invention, there is provided the aforementioned gene-mutated animal wherein the animal having a gene encoding a mutant protein of the amyloid precursor protein and a high productivity of amyloid β protein is a PS1-mutated mouse.

Brief Description of the Drawing

Fig. 1 is a restriction map of a chromosomal DNA fragment Pα containing exon 8 of mouse presenilin-1 which was obtained by cloning from a mouse genomic DNA library.

Fig. 2 illustrates a scheme of the construction method of plasmid pmX-1 containing a partial region of exon 8 of the mouse presenilin-1 gene which comprises

a region introduced with an OS-2 type mutation by a site-directed mutation technique.

Fig. 3 illustrates a process of preparing a targeting vector.

Fig. 4 illustrates a process of preparing a targeting vector.

Fig. 5 illustrates a process of preparing a targeting vector.

Fig. 6 illustrates a process of preparing a targeting vector.

Fig. 7 illustrates a process of preparing a targeting vector and the structure of the targeting vector pOS-2 neoloxP.

Fig. 8 illustrates results of electrophoresis on 1% agarose gel of the PCR product obtained by mating #2 mouse (male) having OS-2 mutant presenilin-1 gene with F4 of CAG-cre#13 mouse (female), cutting a small piece off from the resulting progeny's tail, obtaining chromosomal DNA from the specimen, and carrying out PCR according to the method described in Example 10. It is shown that mice correspond to 2nd and 4th lanes from the right have no neo expression unit on their chromosomal DNA. In the figure, the leftmost lane shows a molecular weight marker. [A] indicates bands showing neo deficiency on the chromosomal DNA, [B] indicates bands showing that the chromosomal DNA is the wild type, and [C] indicates is bands showing the existence of neo on the chromosomal DNA.

Best Mode for Carrying Out the Invention

A mutant presenilin gene used in the production of the gene-mutated animal of the present invention is a gene encoding a mutant presenilin protein, and as used herein, "mutant presenilin gene" means either of, or both of a mutant presenilin-1 gene or a mutant presenilin-2 gene and "mutant presenilin protein" means either of, or both of a mutant presenilin-1 protein or a mutant presenilin-2 protein. The mutant presenilin gene has the property of increasing the production of amyloid β protein. The gene-mutated animal of the present invention is a mammal transferred with the above-mentioned mutant presenilin gene for example by homologous recombination. The mutation existing in the mutant protein is preferably a result of substitution of an amino acid residue. The number of mutations is not limited, and may preferably be 1.

The full length sequence of a mammal-derived preselin-1 protein is described

in, for example, E. Levy-Lahad, et al., Science, 269, pp.973-977, 1995. The full-length sequences of human and mouse presenilin-1 proteins and examples of DNA sequences that encode the proteins are shown in the sequence listings as SEQ ID NOS: 1 to 4. For example, in the mouse-derived presenilin-1, mutation sites may preferably be one or more sites selected from No. 79, No. 82, No. 96, No. 115, No. 120, No. 135, No. 139, No. 143, No. 146, No. 163, No. 209, No. 213, No. 231, No. 235, No. 246, No. 250, No. 260, No. 263, No. 264, No. 267, No. 269, No. 280, No. 285, No. 286, No. 290, No. 318, No. 384, No. 392, No. 410, No. 426, and No. 436.

More preferable mutations are one or more mutations selected from the group consisting of A79V, V82L, V96F, Y115H, Y115C, E120K, E120D, N135D, M139V, M139T, M139I, I143F, I143T, M146L, M146V, H163Y, H163R, G209V, I213T, A231T, A231V, L235P, A246E, L250S, A260V, C263R, P264L, P267S, R269G, R269G, R269H, E280A, E280G, A285V, L286V, S290C, E318G, G384A, L392V, C410Y, A426P, and P436S in the amino acid sequence of the presenilin-1 protein, more preferably in the amino acid sequence of the mouse-derived presenilin-1 protein. Among these mutations, the mutation wherein the amino acid at position 213 is substituted with another amino acid (referred to in some cases as "OS-2 type mutation" in the specification) is a particularly preferable mutation. For example, a mutation wherein isoleucine at position 213 is substituted with an amino acid other than isoleucine, or a mutation wherein isoleucine at position 213 is substituted with threonine is most preferable.

The full-length sequence of a mammal-derived preseline-2 protein is described in, for example, Science, 269, pp. 973-977, 1995. Position 141 and/or position 436 are preferable mutation sites, and in the mouse-derived sequence N141I and/or M239V are more preferable. One or more mutations may exist in either of presenilin-1 protein or presenilin-2 protein, or both of the proteins.

The gene-mutated animal of the present invention is characterized by having the above mutant presenilin-1 gene and/or mutant presenilin-2 gene on its chromosomal DNA. The gene-mutated animal is not limited so far that the animal is a mammal and a kind of the animal is not particularly limited. For example, a rodent may suitably be used. A mouse is particularly preferred. The gene-mutated animal of the present invention can be produced by constructing a plasmid using a

DNA having a sequence of about 10kbp comprising a mutant presenilin gene, and then transferring the plasmid into an embryonic stem cell and thereby causing homologous recombination intracellularly.

The gene-mutated animal of the present invention is characterized in that the amino acid mutation occurs mostly at only one position due to the transfer of the aforementioned mutant presenilin-1 and/or presenilin-2 gene by homologous recombination. In the case of a so-called "trans-genic animal", a DNA sequence comprising a mutant portion is inserted randomly into chromosomal DNA, and tens of copies of a repeated sequence are inserted at plural sites. The gene-mutated animal of the present invention can avoid the problems, and it is possible to accurately analyze pathology of Alzheimer's disease at genetic level. Where a DNA comprises a marker or the like is transferred to the gene-mutated animal of the present invention, the animal may have a site of the marker and a sequence for insertion of the marker. For example, for insertion at a site capable of being cleaved with Sau3AI, one nucleotide can be substituted, and such substitution can be verified by cleaving a PCR product with Sau3AI, followed by subjecting the fragments to electrophoresis or the like.

The gene-mutated animal of the present invention has a characteristic feature of producing amyloid β protein in a larger amount in comparison with a normal animal due to the genetic mutation. An increased amount of amyloid β protein achieved by the gene-mutated animal of the present invention is not particularly limited, and the amount may preferably be sufficient for recognition of a substantial difference in the evaluation of degrees of memory disorder, pathological observations, and various neural disorders as compared to a normal animal.

DNAs, plasmids, cell cultures, and embryos of mammalian cells provided by the present invention are characterized to have a mutant presenilin-1 gene and/or a mutant presenilin-2 gene. For example, a cDNA or a full-length chromosomal DNA of a mutant presenilin-1 gene encoding the mutant presenilin-1 protein, preferably an OS-2 type mutant presenilin-1 protein, or the DNA sequence comprising one or more mutation sites; a plasmid comprising a DNA being the above cDNA or full length chromosomal DNA, or the above DNA comprising one or more mutation sites, which is further introduced with an Sau3AI site; a chromosomal DNA comprising exon 8 of a

mutant presenilin-1 gene encoding an OS-2 type mutant presenilin-1 protein fall within the present invention. Further, the present invention encompasses the above gene or the DNA which further comprises one or more, preferably 1 to 20, more preferably 1 to several substitutions of bases.

Examples of DNAs and plasmids of the present invention include, for example:

1) a DNA comprising a mutant presenilin-1 gene encoding a mutant presenilin-1 protein wherein isoleucine at position 213 of the presenilin-1 protein is substituted with threonine, or a plasmid comprising said DNA;

2) a DNA comprising a mutant presenilin-1 gene wherein a DNA nucleotide sequence encoding amino acids around position 213 of the amino acid sequence of a mutant presenilin-1 protein is the following sequence:
5'-TGTGGTCGGGATGAT M GCCA N CCACTGGAAAGGCCC-3'
wherein N represents a nucleotide other than T and M represents T or C, or a plasmid comprising said DNA;

3) a DNA comprising a mutant presenilin-1 gene wherein a DNA nucleotide sequence encoding amino acids around position 213 of the amino acid sequence of an OS-2 type mutant presenilin-1 protein is the following sequence:
5'-TGTGGTCGGGATGAT M GCC XYZ CACTGGAAAGGCCC-3'
wherein M represents T or C, XYZ represents a codon as triplet bases encoding an amino acid other than isoleucine, or a plasmid comprising said DNA;

4) Any one of the DNAs or plasmids comprising said DNAs according to the aforementioned 1) to 4) wherein a Sau3AI restriction site is introduced;

5) a DNA or a plasmid comprising said DNA wherein a Sau3AI restriction site is introduced into a sequence comprising the full-length of a cDNA or a chromosomal DNA of a mutant presenilin-1 gene encoding a mutant presenilin-1 protein wherein isoleucine at position 213 is substituted with threonine in an amino acid sequence of presenilin-1 protein, or into a mutated portion of said sequence,;

6) a DNA comprising exon 8 of a mutant mouse presenilin-1 gene encoding an OS-2 type mutant presenilin-1 protein and a neomycin expression unit flanked by loxP, or a plasmid comprising said DNA; and,

7) a DNA comprising exon 8 of a mutant presenilin-1 gene encoding a mutant

presenilin-1 protein wherein isoleucine at position 213 is substituted with threonine in an amino acid sequence of presenilin-1 protein and a neomycin expression unit flanked by loxP, or a plasmid comprising said DNA. However, the scope of the invention is not limited to these specific examples.

The embryos or the cells provided by the present invention includes an embryo or a cell into which the above plasmid, e.g. a plasmid comprising a PRL-104 or PRL-105 nucleotide sequence is transferred. Preferable cells of the present invention include those transferred with a gene encoding a mutant presenilin protein which comprises a mutation at position 213 of the amino acid sequence of presenilin-1 protein by homologous recombination using the aforementioned plasmid. Sorts of the embryos or cells are not limited so far that they are derived from a mammal, and those derived from a rodent, preferably a mouse may be used.

Production of the gene-mutated animal

After the DNA encoding a mutant of human presenilin is obtained, the presenilin gene mutated animal of the present invention can be produced according to the process described below. An example will be explained wherein a mouse is used as an mammal and the human mutant human presenilin-1 gene is used as the mutant human presenilin gene. However, the gene-mutated animal of the present invention is not limited to those produced by using these materials. Further, this method is one example of the method of production of the gene-mutated animal of the present invention and the method of the present invention is not limited to the following method. By referring to the general method described below and specific methods described in the examples, and by suitably modifying or altering these methods as required, a person skilled in the art can readily produce the gene-mutated animal of the present invention.

In order to prepare a probe for use in the PCR method, a DNA fragment, which comprises a site for mutation in exon 8 of a presenilin-1 gene deriving from an animal to be used for the production, is obtained from a mouse genomic DNA library. A mouse genomic DNA library of any strain may be used, including a mouse genomic library from mouse 129 strain described in the examples. Where a mouse is used as an animal for introduction of mutation, exon 8 of mouse presenilin-1 gene is used.

Where other sort of animal is used, it is necessary to select an appropriate segment.

After of the DNA fragment prepared by the above process is labeled (^{32}P) by random priming, screening of the genomic library is performed using the labeled probe, and a chromosomal DNA fragment comprising exon 8 of the presenilin-1 gene is then cloned. A portion for mutation in exon 8 of the cloned presenilin-1 gene is further subcloned, and then a mutation is introduced.

A targeting vector is constructed which comprises the chromosomal DNA comprising exon 8 of the mouse presenilin-1 gene into which the mutation was introduced. As a selective marker, neo expression unit is introduced into the targeting vector to facilitate that cells whose chromosome is not introduced with the vector are killed by the addition of G418 (an antibiotic) to a medium. After the targeting vector is introduced into an ES cell by means of electroporation or by another method for gene transfer into a cell, the ES cells are cultured in the presence of G418 and colonies formed are collected. Each of the colonies obtained is divided into two portions. One portion is preserved by culturing, sub-culturing, or freezing. The other portion is used to investigate ES cells into which a desired mutation in exon 8 of the mouse presenilin-1 gene is introduced by homologous recombination. , are examined. The preserved portion of the colony of the ES cells with the desired mutation introduced is taken and used in the process below.

From a pregnant mouse, an embryo at the 8-cell stage is removed. The embryo is sprinkled with about 20 of the above-mentioned preserved ES cells, and then introduced into the uterus of a pseudopregnant female mouse. From among the born young, mice of chimeric coat color are selected. The chimeric mouse is mated with a mouse C57BL/6 strain, and a mouse having the desired mutation can be obtained by the selection of those with agouti coat color from among the born young. The resulting mouse is heterozygote in relation to the presenilin-1 gene introduced with the mutation, whereas the presenilin-1 gene on the other chromosome is a wild type with no mutation.

As starting materials for preparing the probe for the cloning of the chromosomal DNA comprising exon 8 of the mouse presenilin-1 gene from the mouse genomic DNA library, a cDNA of a presenilin-1 gene, which is derived from a mammal other than mouse or human and whose nucleotide sequence has been known, may be

used as well as those specifically mentioned in the Examples. As methods for obtaining the DNA fragment used as the probe, a method for a large scale preparation of a plasmid, which comprises a mouse chromosomal DNA comprising a region corresponding to exon 8 of the mouse presenilin-1 gene in chromosomal DNA, or a cDNA of a presenilin-1 gene derived from a mammal other than mouse or human or the like whose nucleotide sequence has been know, can be applied as well as amplification by PCR described in the Examples. Furthermore, after the plasmid is cleaved by restriction enzymes, a desired DNA fragment can be obtained by separating a portion used as the DNA fragment by means of agarose gel electrophoresis and the like.

As a method for labeling the DNA fragment, methods such as those utilizing PCR in the presence of ^{32}P-dNTP may be used as well as the random priming method described in the Examples. Further, labeling may be introduced by PCR or random priming using a pre-labeled oligodeoxynucleotide as a primer. For the labeling, chemiluminescence using Biotin-Avidin or alkalinephosphatase or the like may also be used, as well as radioisotopes explained in the examples. An RNA fragment labeled by using T3 or T7 RNA polymerase may also be used as a probe. Various methods for preparing a probe are known other than those mentioned above, and a desired probe may be obtained by any method.

For introducing a desired mutation in a DNA, methods specifically described in the Examples can be applied. In addition, a plasmid derived from a bacteriophage such as M13 or a plasmid duplicated using ung$^-$ Escherichia coli is bound complimentarily with an oligodeoxynucleotide synthesized for introducing a mutation at a desired mutation site (bases of the site to be introduced with the mutation are not complimentary), and the resulting complex is used as a primer to prepare a heteroduplex DNA plasmid using a DNA polymerase, and then Escherichia coli (ung$^+$) is transformed with the resulting plasmid to obtain a plasmid having a desired mutation. Another method (cassette method) is applied for to obtain a plasmid having a desired mutation, which comprises the steps of synthesizing two oligodeoxynucleotide, which have modified bases to introduce a desired mutation, and are capable of annealing in a mutually complimentary manner and designed to give restriction enzyme sites at both terminals, and ligating the oligodeoxynucleotide to a

plasmid for introduction of a mutation using DNA ligase. By appropriately modifying or altering the above methods depending on a purpose, the object may sometimes be more effectively achieved. In addition, as method for introducing a mutation, various methods available in the art are known, and accordingly, any method can be applied to achieve the object.

The targeting vector may preferably comprise a selective marker expression unit as an essential element which comprises a mouse chromosomal DNA fragment introduced with a mutation, a DNA fragment encoding a selective marker, a promoter for controlling transcription thereof, and a terminator. The mouse chromosomal DNA fragment introduced with a mutation is a necessary portion for causing homologous recombination in the ES cell, and the mouse chromosomal DNA fragments flanking the position of the mutation at both sides are also necessary. The target vector thus has a DNA fragment in which only the mutated bases are different from a native mouse chromosomal DNA. The length of the fragment may preferably about 10kbp, and generally some degree of lengthening or shortening is permissible. However, where the fragment is too short, frequency of homologous recombination may sometimes be lowered.

As selective markers, positive selective markers such as neomycin-resistant gene and hygromycin-resistant gene, and negative selective markers such as thymidine kinase gene of herpes simplex virus and fragment A of diphtheria toxin are known. Any of markers used for cell culture may be used in ES cells. Where a negative selective marker is used, it is necessary to insert the marker outside the mouse chromosomal DNA fragment of the targeting vector. Where a positive selective marker is used, it is necessary to insert the expression unit in an intron in the mouse chromosomal DNA fragment of the targeting vector. When a positive marker is inserted in an exon, the inserted gene generally loses function, and a mouse cannot be sometimes produced which is to be produced for examination of effects of the mutation as an ultimate purpose.

As an ES cell line, cell lines deriving from mouse 129 strain are frequently used. As ES cells deriving from the above mouse strain, ES cells such as D3, CCE, J1, and AB1 may be used as well as R1 described in the Examples. For example, mouse-derived ES cells such as from C57BL/6 mouse strain may also be used other

than those from 129 strain. As methods for the introduction of the targeting vector into ES cells, electroporation as described in the Examples may generally applied. Any method may be used so far that the method is usable for the introduction of a plasmid into a cultured cell line, such as Ca phosphate coprecipitation or a liposome method. When ES cells introduced with the targeting vector are cultured in the presence of a selective marker, ES cells that survive and form colonies are possibly received homologous recombination. As a method for determining whether homologous recombination occurs in the ES cells that form the colonies, PCR is typically used. A DNA fragment, an RNA fragment, synthetic oligodeoxynucleotide, antibody or the like that is usable as a probe may be employed.

After ES cells are mixed with a fertilized egg at an early stage of development and then development is continued, a mouse from a sperm or an ova deriving from the ES cell can be obtained. To mix the ES cells in which homologous recombination occurs with the fertilized egg at an early stage of development, a method explained in the Examples may be applied. In addition, a method may also be applied which comprises the steps of removing a fertilized egg at blastula stage from a pregnant mouse, injecting 10 to 20 ES cells to the egg using an injection pipette, transplanting the treated egg into the uterus of a pseudopregnant mouse, and then continuing development to obtain the young.

The fertilized egg at an early stage of development for the use of mixing with the ES cells may be eggs obtained from any strain of mouse. In order to facilitate determination whether or not ES cells is incorporated into the progeny, it is preferable to use a fertilized egg from a mouse strain that has a coat color different from that of a mouse strain from which the ES cells are derived. For example, the ES cells used in the Examples are of agouti-colored 129-strain and the mouse from which the fertilized egg is derived (C57BL/6) has a black colored coat. Using these materials, it is possible to easily select the young which have cells derived from the ES cells by selecting the young with chimera coat color from among the born young. In this case, the young with high proportion of agouti color are most likely to have germ cells derived from the ES cells. The pseudopregnant mouse may be of any strain of mouse.

The mouse used to obtain a mouse with the desired introduced mutation

through mating with the resulting chimera mouse may preferably a mouse of a strain with a coat color different from that of a mouse of a strain from which the ES cells are derived. Normally a male chimera mouse is mated with a female of a different strain, and if agouti colored young are obtained, the resulting mice have the desired mutation as heterozygous state. Since a mouse possessing an OS-2 type mutant presenilin-1 gene has a neo expression unit flanked by loxP sequences, it is possible to obtain a mouse in which the neo expression unit is removed can be obtained through mating with a trans-genic mouse with a transferred cre gene.

As explained in the prior art of the present specification, it is believed that a mutation of presenilin-1 protein and presenilin-2 protein promotes the formation of senile plaque due to an increase in Aβ42 and thereby triggers the onset of Alzheimer's disease. Among trans-genic mice introduced with a gene encoding the mutant APP causing familial Alzheimer's disease, some mice are reported to produce amyloid deposition in the brain (Games D., et al., Nature, Vol. 373, p.523, 1995, Hsiao K. et al., Science, Vol. 274, p.99, 1996, Sturchler-Pierrat C. et al., Proc. Natl. Acad. Sci. U.S.A. Vol. 94, No. 24, p.13287, 1997). In these trans-genic mice, it is considered that amyloid deposition is induced by the increase of the amount of Aβ production in the brain.

By mating a trans-genic animal which is transferred with a gene encoding a mutant APP and capable of forming amyloid deposition in the brain (the animal may be homozygous or heterozygous with reference to the transferred gene) with a PS1 gene-mutated animal of the present invention (the animal may be homozygous or heterozygous with reference to the transferred gene), a hybrid animal can be produced. The animal is preferably as mouse. For mating, either of the above animals may be male.

A portion of the tail of the progeny is collected and chromosomal DNA is extracted. PCR is conducted by using the extracted chromosomal DNA as a substrate and by using as primers two oligodeoxynucleotides each having a nucleotide sequence designed to flank the mutation site of a gene encoding the mutant APP and two oligodeoxynucleotides having a nucleotide sequence designed to flank the mutation site of the mutant PS1 gene.

It is possible to determine whether or not the gene encoding the APP mutant

and the mutant PS1 gene of the present invention are incorporated in an extracted chromosomal DNA by carrying out agarose gel electrophoresis of a PCR product, and then observing, for example, presence or absence of bands and mobility of the bands in the gel, and examining the band with the mutation by means of hybridization using an oligodeoxynucleotide having a nucleotide sequence comprising the mutation. PCR may be conducted according to the method described in Example 8. Nucleotide sequences of the oligonucleotides used as the PCR primers may be any sequences so long as they are capable of detecting the gene encoding the APP mutant or the mutant PS1 gene. Based on the results of PCR, an animal having both of the genes each in heterozygous state can be obtained by selection of animals having the gene encoding the APP mutant and the mutant PS1 gene of the present invention.

In order to obtain animals having both of the gene encoding APP mutant and the mutant presenilin-1 gene of the present invention each in homozygous state, an individual animal having both of the genes in homozygous state is selected from the young obtained by mating a suitable male and female selected from the animals having both genes in heterozygous state. To confirm possession of the gene encoding the APP mutant in homozygous state, a potion of the tail of the progeny is taken and chromosomal DNA is extracted, and after the cleavage of the extracted chromosomal DNA with restriction enzyme, electrophoresis is conducted using agarose gel or acrylamide gel. The DNA is then blotted onto a membrane filter, and Southern blotting is performed using as a probe an oligodeoxynucleotide having a sequence which enables binding specifically to a gene encoding the APP mutant, and then density of the resulting bands are measured.

Similarly to the above process, possession of the mutant presenilin-1 gene of this invention in a homozygous state can be verified. Oligodeoxynucleotides used as probes in Southern blotting can be used after being labeled with means ordinarily used in Southern blotting such as a radioactive isotope and a fluorescent dye. A mouse having both of the gene encoding the APP mutation and the mutant presenilin-1 gene of the present invention can thus be produced. A hybrid mouse produced by the above method is characterized by higher productivity of amyloid β protein in the brain and promoted amyloid deposition.

Using the gene-mutated animal, the cells transferred with the mutant

presenilin gene, the plasmid comprising the mutant presenilin gene and the like, it is possible to screen substances useful for preventive and/or therapeutic treatment of Alzheimer's disease and to evaluate their utility. Accumulation of amyloid β in a healthy mammal progresses very slowly, whereas the gene-mutated animal of the present invention has a characteristic feature of higher productivity of amyloid β. Therefore, by administering variety of test substances to the gene-mutated animal of the present invention, and comparing the animal with non-administered animals or animals administered with a control substance, it is possible to evaluate substances useful for preventive and/or therapeutic treatment of Alzheimer's disease. A typical example of the evaluation includes a screening of test substances, and conditions, pathological observations, pharmacological tests and the like can be applied as examinations.

Where the cells of the present invention are used, cells are isolated from the animal of the present invention for the use as a primary cell culture, and then the cells can be stabilized and made into a subcultured cell line by immortalizing the cells of primary culture by treatment with a virus or the like, subculturing the cells by isolating a portion of the culture and subjecting to further cultivation in a fresh tissue culture medium. The cells of the present invention encompass the primary cell culture such as nerve cells isolated from the gene-mutated animal, as well as subcultured cells, i.e., so-called cell lines, obtained by subculturing the primary culture. When a nerve cell is used as the cell of the present invention, the cell expresses a large amount of amyloid β protein due to a result of the expression of mutant presenilin-1 protein by the cell. Substance which prevent or delay the nerve cell death related to accumulation of amyloid β can be screened and utility thereof can also be evaluated by adding a test substance to an in vitro culture system of such nerve cells, and comparing, for example, cell survival period or surviving cell number after a certain period of time.

Examples

The present invention will be more specifically explained by way of examples. However, scope of the present invention is not limited to these examples. In the following examples, presenilin-1 gene is occasionally referred to as PS-1.

Example 1: Cloning of Chromosomal DNA containing Exon 8 of Mouse Presenilin-1 (PS-1) Gene

To construct a probe for isolating a chromosomal DNA containing exon 8 of the mouse PS-1 gene, the following two oligodeoxynucleotides were synthesized:

PR-8-U: 5'-GGAATTTTGGTGTGGTCGGGATGAT-3' (25-mer)

PR-8-L: 5'-GGTCCATTCGGGGAGGTACTTGA-3' (23-mer)

PCR was carried out by using these two oligodeoxynucleotides as PCR primers and DNA extracted from 129 SVJ mouse genomic library (Stratagene) to obtain amplified DNA fragment of approximately 130 bp. The fragment was then labeled by random priming method in the presence of ^{32}P-dCTP and then used as probes for screening of the 129 SVJ mouse genomic library. The resulting positive phage clones were examined and confirmed that they carried the desired chromosomal DNA including exon 8 of the mouse PS-1 gene. The cloned chromosomal DNA was designated as P α and subjected to restriction mapping (Figure 1).

Example 2: Construction of Plasmid for Introducing Mutation

DNA was extracted from the cloned phage carrying P α and cleaved with Sal I, and then subjected to electrophoresis on 1.0 % agarose gel to collect P α. After the cleavage with Pst I and Xba I, the product was subjected to electrophoresis on 1% agarose gel to collect a DNA fragment of approximately 600 bp including a nucleotide sequence encoding isoleucine at position 213 of mouse PS-1. The resulting DNA fragment was designated as X-1. X-1 was ligated using T4 ligase to the plasmid pBluescript II KS+ (Stratagene) which was cleaved beforehand with PstI and Xba I, and then used to transform Escherichia coli to obtain plasmid pX-1.

Example 3: Introduction of OS-2 Type Mutation

An OS-2 type mutation and a Sau3A I restriction site were newly introduced into the plasmid pX-1 using the following two oligodeoxynucleotides PRL-104 and PRL-105. Both PRL-104 and PRL-105 were 36-mers and complementary to each other:

PRL-104: 5'-TGTGGTCGGGA TGATC* GCCA C CCACTGGAAAGGCCC-3'

PRL-105: 5'-GGGCCTTTCCAGTGG G TGGCG* ATCATCCCGACCACA-3'

(The underlined base is changed from a wild-type base to introduce the OS-2 type mutation, i.e., T for PRL-104 and A for PRL-105 in wild types. Asterisked bases are changed from wild-type bases to introduce the Sau3A I site, i.e., T for PRL-104 and A for PRL-105 in wild types.)

The introduction of the mutation was carried out by using QUICK CHANGE SITE-DIRECTED MUTAGENESIS KIT (Strategene) according to the manufacturer's protocols. Sequencing of the product verified that the mutation was correctly introduced. X-1 bearing the mutation was designated as mX-1, and the plasmid carrying mX-1 was designated as p mX-1 (Figure 2).

Example 4: Construction of Chromosomal DNA Comprising OS-2 Type Mutation

P α including exon 8 of the mouse PS-1 obtained in Example 1 was cleaved with Nco I, and then treated with T4 DNA polymerase in the presence of four types of dNTPs to form blunt ends. The resulting fragment was further cleaved with Asp718 I and then subjected to electrophoresis on 1 % agarose gel to collect an approximately 5-kbp DNA fragment including exon 8. This fragment was ligated using T4 DNA ligase to the plasmid pBluescript II KS+ which was cleaved beforehand with Sam I and Asp718 I, and then transformed into Escherichia coli to obtain a plasmid pSB-0. The plasmid pSB-0 was completely cleaved with Xba I, followed by partial digestion with Pst I. Plasmid pmX-1 was cleaved with Xba I and Pst I and subjected to electrophoresis on 1 % agarose gel to collect mX-1. The mx-1 was ligated to the Pst I fragment using T4 DNA ligase, and then used to transform Escherichia coli. The colonies of transformed E. coli were screened to select a colony carrying a plasmid in which the X-1 portion in the plasmid pSB-0 was replaced with mX-1. The plasmid collected was designated as pmSB-0 (Figure 3).

Separately, P α was cleaved with BamH I and Sal I and subjected to electrophoresis on 1% agarose gel to collect an approximately 7-kbp DNA fragment including exon 8. This fragment was ligated using T4 ligase to the plasmid

pBluescript II KS+ which was cleaved beforehand with BamH I and Sal I, and then used to transform E. coli to obtain plasmid pSB-1. The plasmid pmSB-0 was cleaved with Nco I and treated with T4 DNA polymerase in the presence of four types of dNTPs to form blunt ends. The resulting fragment was further cleaved with Xba I and subjected to electrophoresis on 1% agarose gel. An approximately 2.2-kbp DNA fragment XN including exon 8 was collected, and then the fragment and the plasmid pSB-1 were cleaved with Xba I and Pst I, and then the products were subjected to electrophoresis on 1 % agarose gel. The collected approximately 2.3-kbp DNA fragment PX not including exon 8 was ligated using T4 DNA ligase. The ligated fragment was further ligated using T4 DNA ligase to the pBluescript II KS+ which was cleaved beforehand with Xba I, blunt-ended with T4 DNA polymerase in the presence of four types of dNTPs, re-ligated using T4 DNA ligase, and cleaved with Sma I and Pst I. The resulting plasmid was subsequently transformed into E. coli. The colonies of transformed cells were screened to obtain plasmid pmSB-0' carrying only one DNA fragment in which DNA fragments XN and XP were ligated at the Xba I site (Figure 4).

Example 5: Construction of Targeting Vector Backbone

To introduce an Eag I site into the Xba I site in plasmid pmSB-0', an oligodeoxynucleotide having the following sequence was synthesized:

5'-CTAGACGGCCGT-3' (12 mer)

This oligodeoxynucleotide is capable of annealing via a nucleotide sequence having complementarity at the portion of CGGCCG, and forming the following sequence after introduction at a site cleaved with Xba I.

5′ – T C T A G A C G G C C G T C T A G A – 3′
3′ – A G A T C T G C C G G C A G A T C T – 5′
Xba I *Eag* I *Xba* I

After the plasmid pmSB-0' was cleaved with Xba I, the above deoxynucleotide

was added to the product and ligated using T4 DNA ligase, and then used to transform E. coli to obtain a plasmid pmSB-0'eag in which the Eag I site was inserted into the Xba I site of the plasmid pmSB-0'. After cleavage of pmSB-0'eag with Nco I and Sal I, resulting fragments were subjected to electrophoresis on 1% agarose gel to collect an approximately 5.3-kbp DNA fragment SN including exon 8. Separately, plasmid pSB-1 was cleaved with BamH I and Nco I and then subjected to electrophoresis on 1% agarose gel to collect an approximately 2-kbp DNA fragment NB not containing exon 8. The fragments SN and NB were ligated using T4 DNA ligase and treated with BamH I and Sal I to obtain a DNA fragment in which both DNA fragments were ligated at the Nco I site. This DNA fragment was further ligated to pBluescript II KS+ using T4 NDA ligase and then used to transform E. coli to obtain a plasmid pA (Figure 5), wherein the pBluescript II KS+ was cleaved beforehand with Not I, blunt-ended with mung bean nuclease, re-ligated using T4 DNA ligase to break the Not I site and the Eag I site overlapping with the site, and cleaved with BamH I and Sal I.

Example 6: Construction of Targeting Vector

Plasmid pPNT (Victor L. J. et al., Cell Vol. 65, p.1153, 1991) was cleaved with Xho I and BamH I and then treated with T4 DNA polymerase to form blunt ends and subjected to electrophoresis on 1 % agarose gel. The collected approximately 1.7-kbp DNA fragment containing a neo expression unit was ligated using T4 DNA ligase to the plasmid pBS246 (GIBCO BRL) which was cleaved beforehand with BamH I and treated with T4 DNA polymerase to form blunt ends, and then used to transform E. coli to obtain a plasmid pBS246neo. The plasmid was cleaved with Not I and then subjected to electrophoresis on 1% agarose gel to collect an approximately 2-kbp DNA fragment including the neo expression unit flanked by loxP sequences. The obtained DNA fragment was ligated using T4 DNA ligase to the plasmid pA which was cleaved beforehand with Eag I, and then used to transform E. coli. Colonies of transformed cells were screened to obtain plasmid pB in which the neo gene and the PS-1 gene were oriented in the same direction (Figure 6).

After cleavage of the plasmid pB with BamH I and Sal I, the resulting fragments were subjected to electrophoresis on 1% agarose gel to collect a DNA

fragment C containing the OS-2 type mutation and the neo expression unit flanked by loxP sequences. Similarly, P α was cleaved with Sal I and BamH I, and the resulting DNA fragment of approximately 6.5 kbp was subcloned into pBluescript II KS+ to construct a plasmid pSB-2, which was then cleaved with Hind III and BamH I and subjected to electrophoresis on 1% agarose gel to collect a DNA fragment D of approximately 4kbp. DNA fragments C and D were ligated using T4 DNA ligase, and then the product was cleaved with Hind III and Sal I to obtain a DNA fragment in which C and D were ligated at the BamH I site. The obtained DNA fragment was further ligated using T4 DNA ligase to the pBluescript II KS+ which was cleaved beforehand with Hind III and Sal I, and then used to transform E. coli to obtain a targeting vector pOS-2neoloxP (Figure 7).

Example 7: Introduction of Targeting Vector into ES Cells

Hereinafter in the examples, culture was carried out in an incubator at 37 °C under 5% CO_2. The targeting vector was introduced by electroporation into ES cells (R1) which were maintained in DMEM medium supplemented with 15% FBS and 10^3 units/ml LIF (ESGRO) (the DMEM medium is hereinafter abbreviated as ES medium). Culture medium was replaced with fresh ES medium one day before electroporation, and the R1 cells were collected and washed with electroporation buffer (20 mM HEPES, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM dextrose). R1 cells (10^7 cells) were mixed with 25 μg of the targeting vector pOS-2neoloxP, which was linearized using Not I, and 0.8 ml electroporation buffer in an electroporation cuvette. After 1 to 2 minutes, pulses were applied to the cells using Bio-Rad GenePulser (Bio-Rad) under pulse conditions of 240 V and 500 μF. The ES cells were collected by centrifugation and suspended in 30 ml ES medium. The ES cell suspension (2 ml) was put in each 10ml culture dish in which feeder cells were put in 8 ml ES medium. G418 (titer, 150 μg/ml) was added to the culture after 12 to 18 hours, followed by one-week culture. As the feeder cell, a fibroblast established by the present inventors was used which was isolated from an embryo of 12 to 13 days obtained by mating a HS1 knockout male mouse (I. Taniuchi et al., EMBO J. vol. 14, p. 3664, 1995) with an ICR female mouse of wild-type.

Example 8: Isolation of ES Cells with Homologous Recombination

Colonies of ES cells that were formed in Example 7 by one-week cultivation after the addition of G418 were collected. Each colony was divided into two portions. One portion was subjected to further cultivation. For selection of clones in which homologous recombination occurred, the other portion was washed with PBS, treated with Proteinase K, and then chromosomal DNA was collected and subjected to PCR to select clones. Nucleotide sequences of the synthetic primers used in PCR reaction were as follows.

Prsn1-2: 5'-CCCAACTCTATTTCTACCCTCGTTCATCTG-3'

(nucleotide sequence outside the targeting vector constructed)

PKG-1: 5'-TAGTGAGACGTGCTACTTCCATTTGTCACG-3'

(nucleotide sequence in the neo expression unit)

PCR reaction was carried out for 35 cycles under the following conditions: 30 seconds at 93 °C, 1 minute at 60 °C, and 3 minutes at 68 °C per cycle. The PCR product was analyzed by 1 % agarose gel electrophoresis to identify a positive clone which gave a band at an expected position. The clone evaluated as positive was further subjected to PCR using oligodeoxynucleotides PRL-101 and PRL-102. The resulting PCR product was cleaved with Sau3A I and then subjected to electrophoresis on 2% agarose gel. Introduction of the mutation was verified by split bands, and ES cells in which desired homologous recombination occurred were selected. Nucleotide sequences of PRL-101 and PRL-102 were as follows.

PRL-101: 5'-TGCTGGAGGAAAATGTGTTATTTAAGAGCA-3'

PRL-102: 5'-TACTGAAATCACAGCCAAGATGAGCCATGC-3'

Example 9: Production of Knockin Mouse

ES cells verified to have homologous recombination were further cultured for 4 days and then treated with trypsin to separate one another. An eight-celled embryo was taken from a BDF1 female mouse which was mated with a BDF1 male mouse, and its zona pellucida was then removed. The ES cells separated from one another were attached to the naked embryo (20 ES cells per 8-celled embryo). Th treated embryo was transferred into the uterus of a pseudopregnant female mouse and embryonic development was continued to produce chimeric mice. The resulting

chimeric male mouse was mated with a C57BL/6 female mouse. From among their progeny, mice with agouti color were chosen. A portion of the tail was excised, and chromosomal DNA was extracted from each sample. PCR was carried out using PRL-101 and PRL-102, and the PCR product was cleaved with Sau3A I and then subjected to electrophoresis on 2% agarose gel. The presence of cleaved bands was examined to verify that the selected progeny possessed the OS-2 type mutation. One male mouse was chosen from the verified mice and designated as #2.

Example 10

The knockin mouse #2 obtained in Example 9 has the heterozygous neo expression unit flanked by loxPs deriving from the targeting vector. This mouse #2 (male, about 4 months old) was mated with a F4 female of CAG-cre#13 transgenic mouse (2 months old in which transferred cre gene is heterozygous state, K. Sakai et al., Biochem. Biophys. Res. Commun. 217:318, 1997). PCR was carried out using oligodeoxynucleotides PRL-100, PRL-102 and PGK-1 under the conditions described in Example 8. A mouse from which the neo expression unit was removed was chosen as an OS-2 mutated knockin mouse without the neo expression unit (Figure 8). This mouse was heterozygous with reference to OS-2 type mutation, and had one loxP. Nucleotide sequences of PRL-100, PRL-102 and PGK-1 used for the PCR were as follows.

PRL-100: 5'-GGT CCA TCC CAG CTT CAC ACA GAC AAG TCT-3'

PRL-102: 5'-TAC TGA AAT CAC AGC CAA GAT GAG CCA TGC-3'

PKG-1: 5'-TAG TGA GAC GTG CTA CTT CCA TTT GTC ACG-3'

Industrial Applicability

The gene-mutated animal of the present invention has a mutated presenilin-1 gene and high productivity of amyloid β due to the gene in comparison with a normal animal without the mutation, and hence the animal exhibits symptoms of Alzheimer's disease through early cell-death or deciduation of neurons in the cerebral hippocampus. Therefore, screening of substances useful for preventive and/or therapeutic treatment of Alzheimer's disease and evaluation of usefulness thereof can be conducted by using the gene-mutated animal of the present invention.